Dissecting host-pathogen interactions of *Caenorhabditis elegans* and *Agrobacterium tumefaciens*

Research Thesis

By
Anthony W. Kiragu
The Ohio State University
April 2013

Project Advisors:
Helen M. Chamberlin, PhD, Department of Molecular Genetics
Chad A. Rappleye, PhD, Department of Microbiology, Center for Microbial Interface Biology
Birgit E. Alber, PhD, Department of Microbiology

Thesis Committee:
Dr. Helen M. Chamberlin
Dr. Chad A. Rappleye
Dr. Birgit E. Alber

Abstract

DNA transformation is a common tool in genetic research and engineering. In the model organism *Caenorhabditis elegans*, DNA transformation has primarily been through microinjection of the DNA into the syncytial gonad. This ensures the introduced DNA is passed down to the offspring. In an experiment to find alternative methods of genetic transformation, *Agrobacterium tumefaciens* was used as a candidate for the transfer of DNA from the bacteria to the eukaryotic cell nucleus. *Agrobacterium* is well known for its natural capability of trans-kingdom DNA transfer and can transform virtually any living cell. The bacterium has both transformation abilities and infection abilities. While we are working to develop the transformation abilities, we observed an unusual phenomenon in the intestine of worms cultured with the bacteria. Adult worms that were fed *A. tumefaciens* exhibited abnormal fluorescence in the intestinal cells. We are investigating it by setting up two experiments to study the viability and fluorescence difference between wild type and mutants deficient for any intestinal fluorescence. These experiments are designed to test whether the observed abnormal fluorescence results from induction or alteration of the pathway that mediates normal *C. elegans* intestinal autofluorescence, or whether another mechanism is involved. To test this hypothesis, a comparative experiment between wild-type worms and mutants for *glo-1* (lacks autofluorescent and birefringent gut granules) and *glo-4* (no autofluorescent granules in intestinal cells) genes was conducted. For worms cultured in *A. tumefaciens*, it is evident that the fluorescence observed is ancillary to the autofluorescence and present in *glo* mutants, and indication of causality. As the bacterium have virulent effects on the worms, we set up an experiment to define the level of fatality on the worms or resistance by the mutants. Wild type and mutants are being cultured with *A. tumefaciens* and *Escherichia coli* OP50 in this experiment. The long-term goal of this work is to better understand host pathogen interactions, and to optimize the use of

Agrobacterium for DNA conjugation in *C. elegans*.

Table of Contents

Title Page……..i

Abstract……ii

Table of Contents ……iv

Chapter 1: Introduction……5

Chapter 2: Methods……..….8

Chapter 3: Data Analysis and Results……..……....9

Chapter 4: Discussion……..16

References…….……...18

Dissecting host-pathogen interactions of
Caenorhabditis elegans and *Agrobacterium tumefaciens*

Introduction

The nematode *Caenorhabditis elegans* is one of the ideal model organisms used in genetic studies due to the fast maturation and generation time with easy and cheap maintenance. A lot of research has been done using the organism thereby availing a large database of genome sequence and mutant strains. Most importantly, the ability to generate transgenic animals using *C. elegans* has allowed for the study of expression, localization and function of genes. Two efficient strategies have been developed and are widely used for the genetic transformation of the nematode *C. elegans*, DNA microinjection, and DNA-coated microparticle bombardment (Rieckher *et al.*, 2009). These methods target the syncytial gonad to produce reproducible transgenic lines and avoid germline silencing. However, in many gene expression and regulation experiments, there is a need for single-copy insertion of the DNA to ensure stability of the transgene. This is because microinjection, and sometimes microparticle bombardment, introduces multi-copy extrachromosomal DNA arrays. Because extrachromosomal arrays are semistable, only a fraction of the animals in a transgenic extrachromosomal array line are transformed. In addition, because extrachromosomal arrays can contain hundreds of copies of the transforming DNA, transgenes may be overexpressed, misexpressed, or silenced (Praitis *et al,* 2001).

Single-copy insertion is a suitable solution to the capricious expression of multi-copy extrachromosomal arrays but achieving such integration is a very rare event. Microparticle bombardment and transposon insertion are the two alternatives commonly used. Microparticle bombardment produces low-copy and even single-copy chromosomal insertions. Transposon insertion, while a viable option, proves to be very elusive. In our hands, transgenesis experiments

set up using the popular *Mos1* transposon yielded no transgene insertion, and what we observed as insertion event were false positives – integration of DNA was extrachromosomal. Although microparticle bombardment and microinjection methods have shown positive results, the events are rare even at optimal conditions and numerous trials are usually necessary. An alternative method would be valuable.

To this end, we are attempting to use *A. tumefaciens* as a candidate in the transfer of DNA from the bacterium to the eukaryotic cell nucleus, and integration into the host genome. *Agrobacterium* is well known for its natural capability of trans-domain DNA transfer. Although used mainly for plant genetic engineering, *Agrobacterium* can transform virtually any living cell, from other prokaryotes to yeast and fungi to human cells (Tzvi Tzfira et al., 2004). *A. tumefaciens* is a soil bacterium that causes crown-gall disease, a disease of the roots and stems in many plants. It does so by transferring DNA (T-DNA) from its tumor-inducing (Ti) plasmid to the host cell nucleus and integrating into the host genome and expressing its encoded genes. The T-DNA is delimited by 25-bp direct repeats that flank the T-DNA. These borders are the only cis elements necessary to direct T-DNA processing. Any DNA between these borders will be transferred to a plant cell (Zupan and Zambryski, 1995). For the transfer to occur, *A. tumefaciens* must be positioned adjacent to the target host cell, and various complexes in both the host and bacterium facilitate for the transfer into the nucleus of the host cell. As a proof of principle, we have developed *A. tumefaciens* carrying *rpl-28::gfp* DNA (in its Ti plasmid). These bacteria were introduced into wild-type worms using various methods and observed for any changes. We observed an unusual phenomenon in the intestine of worms cultured with the bacteria. Adult worms that were fed *A. tumefaciens* exhibited abnormal fluorescence in the intestinal cells. However, the fluorescence was not a result of insertion of foreign DNA but rather a result

of the interaction between the host and the bacterium.

C. elegans have intestinal cells and gut granules that have autofluorescent properties. To test whether the observed abnormal fluorescence results from induction or alteration of the pathway that mediates normal *C. elegans* intestinal autofluorescence, *glo* mutant strains that lacked autofluorescence were used to carry out the same experiment. Mutant strain JJ1271 (*glo-1* mutant that lacks autofluorescent and birefringent gut granules) and mutant strain RB811 (*glo-4* mutant with no autofluorescent granules in intestinal cells) were incubated with *Agrobacterium*. The gene *glo-1* is required during embryonic development for normal body morphogenesis in regulating gut granule formation. The protein GLO-1 is required for the biogenesis of gut granules (lysosome-related gut organelles) in *C. elegans* (Hermann *et al*., 2005). GLO-4 is a putative guanine nucleotide exchange factor to GLO-1, and *glo-4* mutant animals have similar gut granule phenotypes as *glo-1* mutant animals (Hermann *et al*., 2005).

Based on preliminary results, a reasonable conclusion was that the fluorescence observed was a consequence of the interaction of the intestinal cells and the bacteria. A hypothesis was then formulated to study any correlation between *Agrobacterium* and the intestinal cells. We hypothesized that absent some elements of the targeted cells (intestinal cells), *Agrobacterium* would have a different effect – perhaps have less virulence towards *C. elegans*. The *glo* genes are expressed in the intestinal cells and are responsible for autofluorescence. Therefore, a study of viability between *glo* mutants and wildtype worms was in order.

Methods

Preparation of LB + spectinomycin plates: Dissolve 8g NaCl, 10g tryptone and 5g yeast extract in 1L of water. Adjust pH with 1M NaOH to pH 7. Add 1% agar for solid medium. Autoclave at 121°C for 20 minutes. Cool to 60°C. Add spectinomycin (250µg/mL) and kanamycin (100 µg/mL). Pour 30mL onto petri dishes.

Preparation of Nematode Growth Medium (NGM) plates: Mix 3 g NaCl, 17 g agar, and 2.5 g peptone in 1L of water. Autoclave for 50 min. Cool flask in 55°C water bath for 15 min. Add 1 mL 1 M $CaCl_2$, 1 mL 5 mg/mL cholesterol in ethanol, 1 mL 1 M $MgSO_4$ and 25 mL 1 M KPO_4 buffer. Using sterile procedures, dispense the NGM solution into petri plates using a peristaltic pump. Fill plates 2/3 full of agar. Leave plates at room temperature for 2-3 days before use to allow for detection of contaminants, and to allow excess moisture to evaporate.

Preparation of E. coli OP5O plates: Using sterile technique, apply approximately 0.05 mL of *E. coli* OP50 liquid culture to small NGM plates.

Preparation of A. tumefaciens plates: 3-5 colonies of *A. tumefaciens* were scraped from a LB + spectinomycin plate and suspended in 1 mL of distilled water. The solution was used to dispense 50 µL onto NGM plates. The plates were left at room temperature to dry.

Obtaining glo mutants: Two mutant strains JJ1271 (*glo-1* mutant) and RB811 (*glo-4* mutant) were obtained from Caenorhabditis Genetics Center at the University of Minnesota.

Selecting and plating worms: To test viability, stage-4 larva (L4s) of wild type and mutant worms were picked from *E. coli* OP50 plates onto *E. coli* OP50 (25 wt, 15 *glo-1* and 20 *glo-4*) and *A. tumefaciens* plates (24 wt, 43 *glo-1* and 18 *glo-4*), two or three par plate. On the second day, the worms were transfer to respective plates to separate them from their offspring. Over a period of several days, the number of worms alive on each plate was recorded.

Imaging worms: Adult worms grown on *E. coli* OP50 and *A. tumefaciens* were placed on slides and observed under a microscope for fluorescence.

Data Analysis and Results

Fluorescence Expression

Imaging the worms, both wild type and mutants, revealed the expression levels of fluorescence after inoculation with *E. coli* OP50 and *Agrobacterium.* Wildtype worms exhibit autofluorescence while inoculated with *E. coli* OP50 (Fig. 1). Although while inoculated with *Agrobacterium*, it was difficult to determine which fluorescence was autofluorescence and which was as a result of *Agrobacterium*, it was evident that there was fluorescence expression levels that at least equal to if not greater than those inoculated with *E. coli* OP50 (Fig. 2).

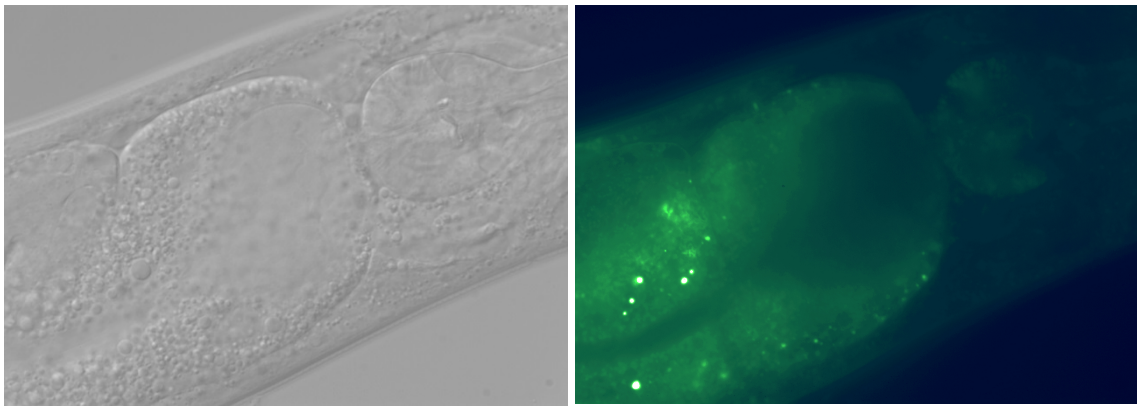

Figure 1: The intestinal-cell region posterior to the pharynx of an adult wildtype worm feeding on *E. coli* plate exhibited autofluorescence under UV light (1000X)

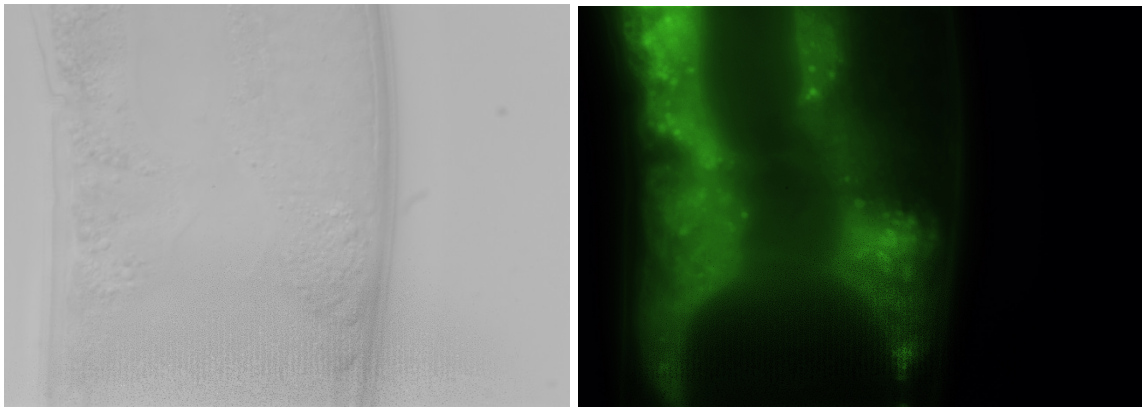

Figure 2: The intestinal-cell region posterior to the pharynx of an adult wildtype worm from an *Agrobacterium* plate exhibited increased fluorescence under UV light (1000X)

Mutant worms for *glo-1* and *glo-4* genes have no autofluorescence in the intestinal cells, and therefore great candidates for defining the expression levels that are directly as a result of *Agrobacterium*. A *glo-1* adult that was inoculated with *E. coli* OP50 had barely any visible fluorescence (Fig. 3) as expected but the opposite was true for the adult inoculated with *Agrobacterium* (Fig. 4).

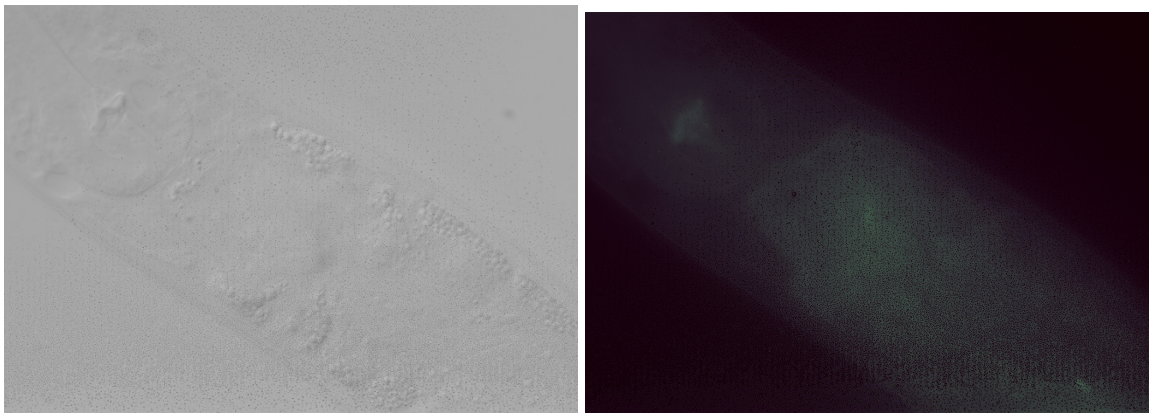

Figure 3: The intestinal-cell region posterior to the pharynx of an adult *glo-1* mutant worm from an *E. coli* plate exhibited very dim fluorescence under UV light (1000X)

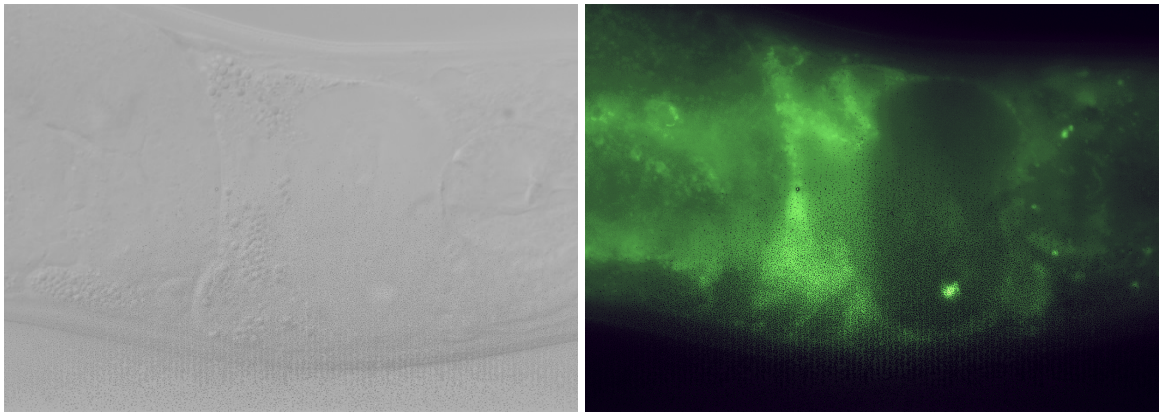

Figure 4: The intestinal-cell region posterior to the pharynx of an adult *glo-1* mutant worm from an *A. tumefaciens* plate exhibited increased fluorescence under UV light (1000X).

Similarly, a *glo-4* mutant adult exhibited fluorescence when inoculated with *Agrobacterium* (Fig. 6) while its *E. coli* counterpart displayed minimal fluorescence (Fig. 5). These results argue that the fluorescence observed in response to growth with *A. tumefaciens* results from a biochemical process independent of the normal autofluorescent process.

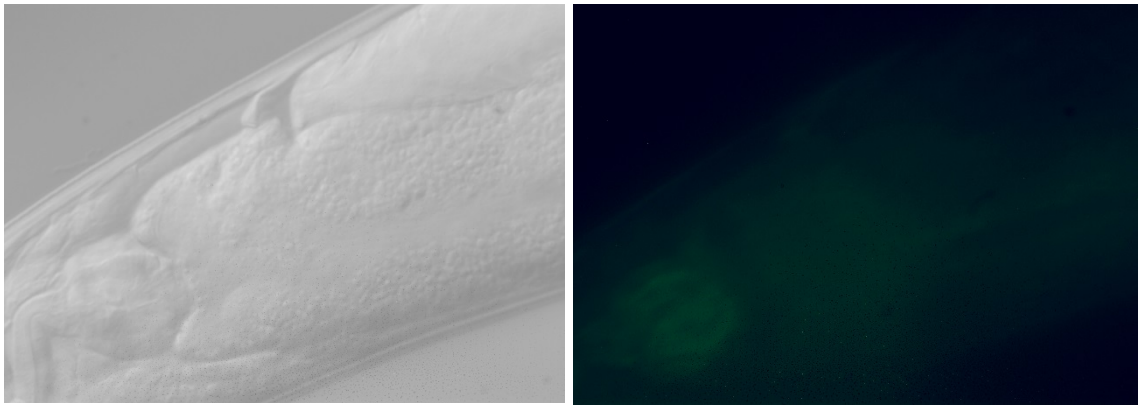

Figure 5: The intestinal-cell region posterior to the pharynx of an adult *glo-4* mutant worm from an *E. coli* plate exhibited very dim fluorescence under UV light (1000X).

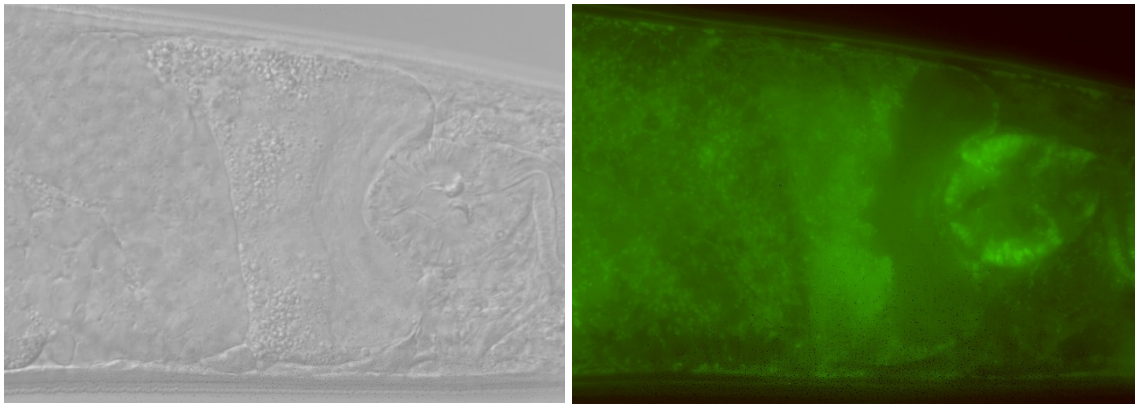

Figure 6: The intestinal-cell region posterior to the pharynx of an adult *glo-4* mutant worm from an *A. tumefaciens* plate exhibited increased fluorescence under UV light (1000X).

Viability Experiment

The number of worms alive were observed and tabulated. These data were used to draw kill curves of the three strains and compared their viability when inoculated with both bacteria. Growth of the three strains on *E. coli* OP50 demonstrated that the wildtype strain (N2) and the *glo-4* mutant strain (RB811) grew at relatively the same pace (Fig. 7) while the *glo-1* mutant strain (JJ1271) experienced less survival (Fig. 8).

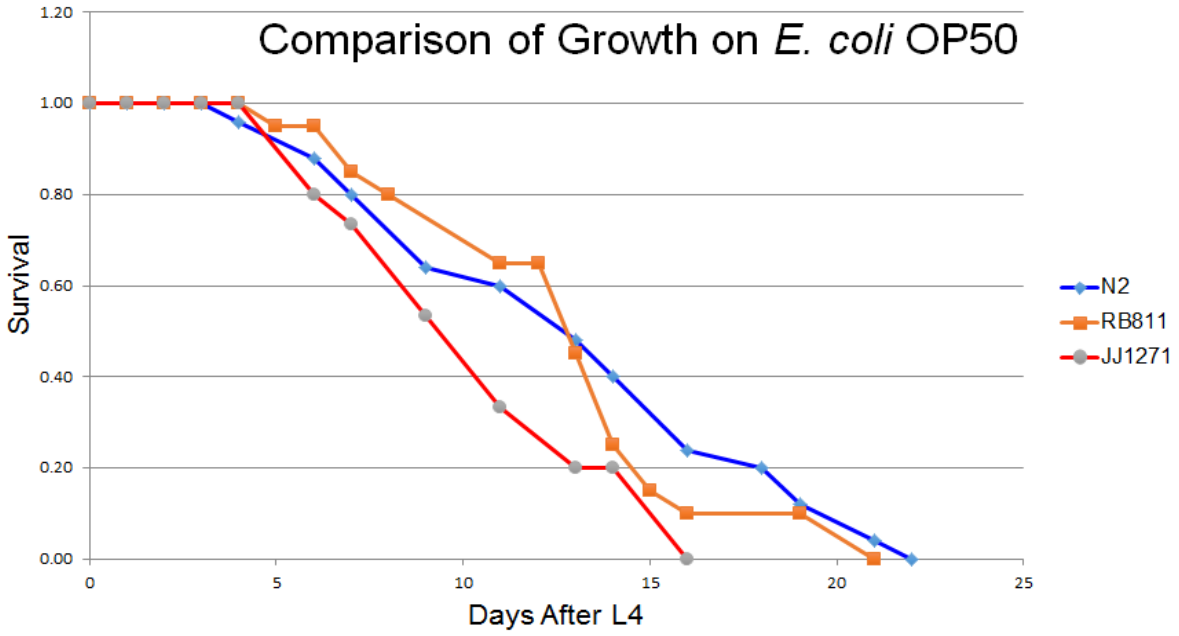

Figure 7: Kill curve of *glo* mutant strains (RB811 & JJ1271) and a wild type strain (N2) from an *E. coli* plate

When the three strains were inoculated with *A. tumefaciens*, they generally had the same viability. There were differences in survival but not statistical significant.

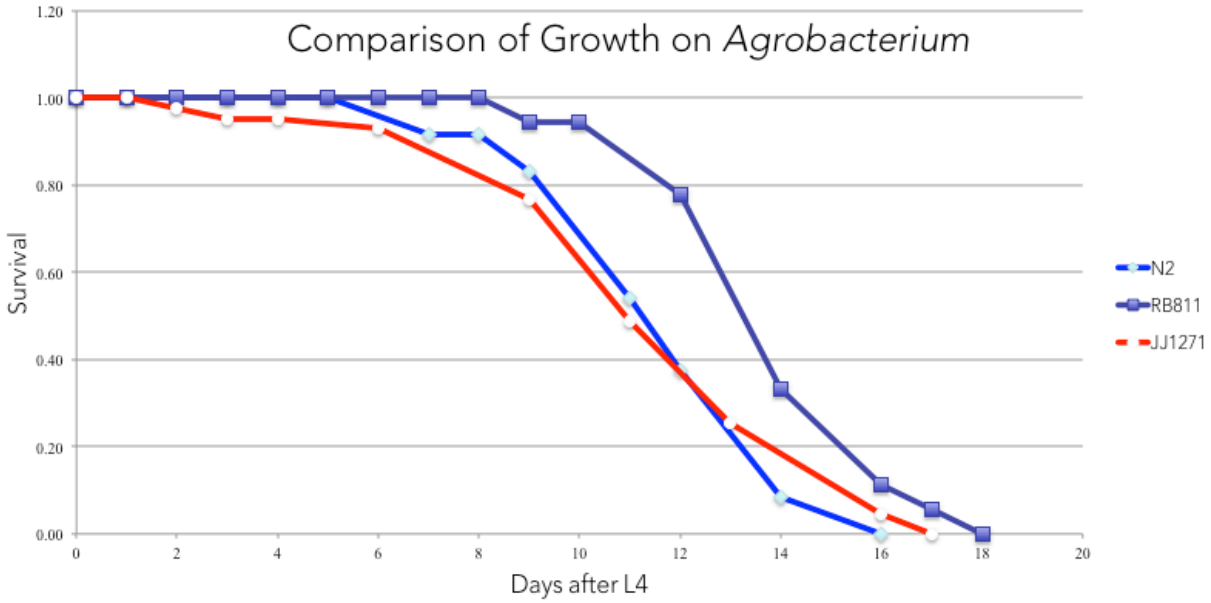

Figure 8: Kill curve of *glo* mutant strains (RB811 & JJ1271) and a wild type strain (N2) from an *Agrobacterium* plate

Within each strain, comparative kill curves were drawn to compare how inoculation with the two bacteria affected survival. For the wild-type strain, there was a six-day reduction in the viability when inoculated with *Agrobacterium* (Fig. 9) but this difference, like the former, is not statistically significant. However, this may be a significant effect of the virulence of *Agrobacterium*.

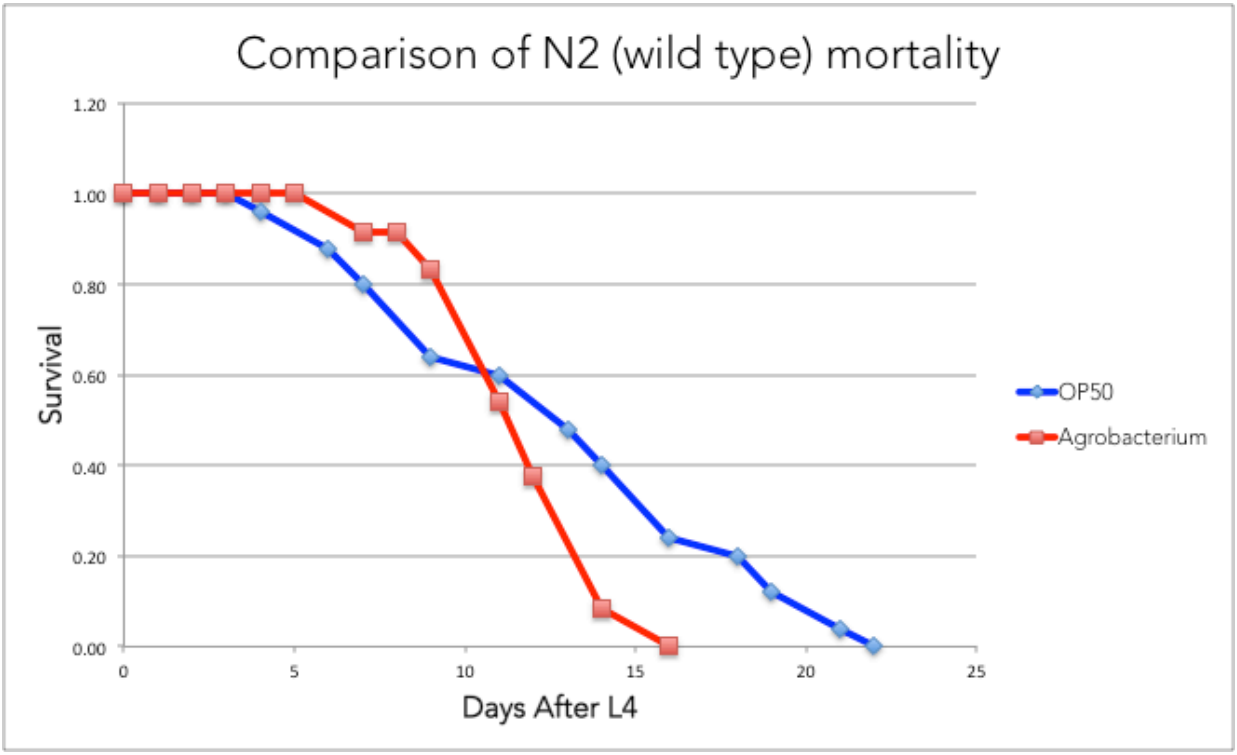

Figure 9: Comparative kill curve of the wildtype strain (N2) on both *E. coli* OP50 and *Agrobacterium* plates (replotted data from Figures 7 and 8).

However, for the mutant strains the viability was about the same. Both strains responded similarly when inoculated with *Agrobacterium* as they did when inoculated with *E. coli* OP50.

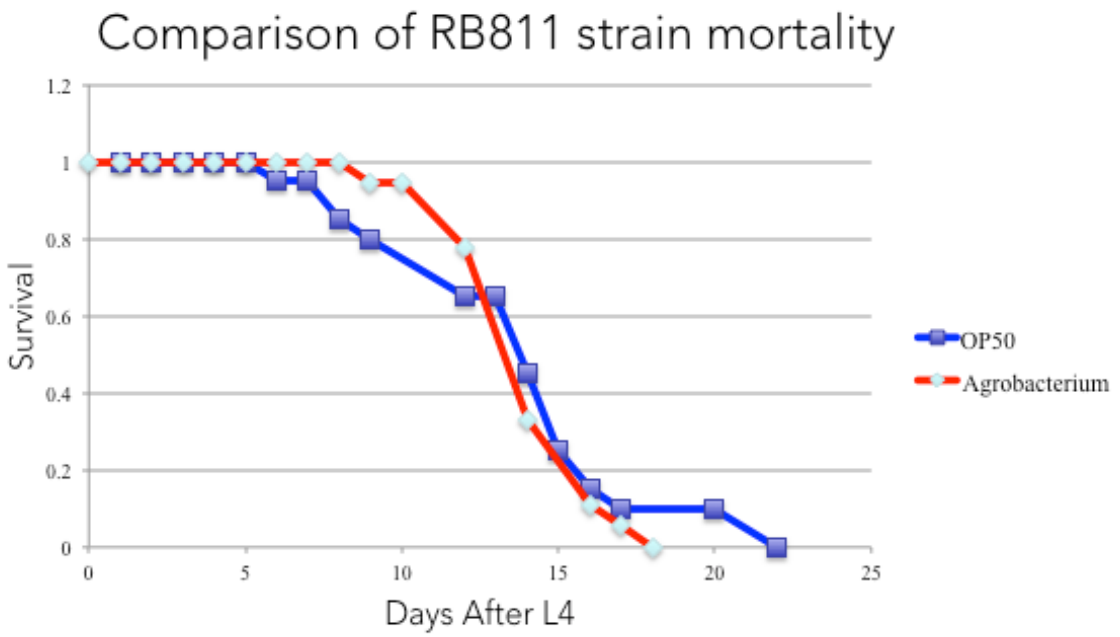

Figure 10: Comparative kill curve of the mutant strain RB811 on both *E. coli* OP50 and *Agrobacterium* plates (replotted data from Figures 7 and 8).

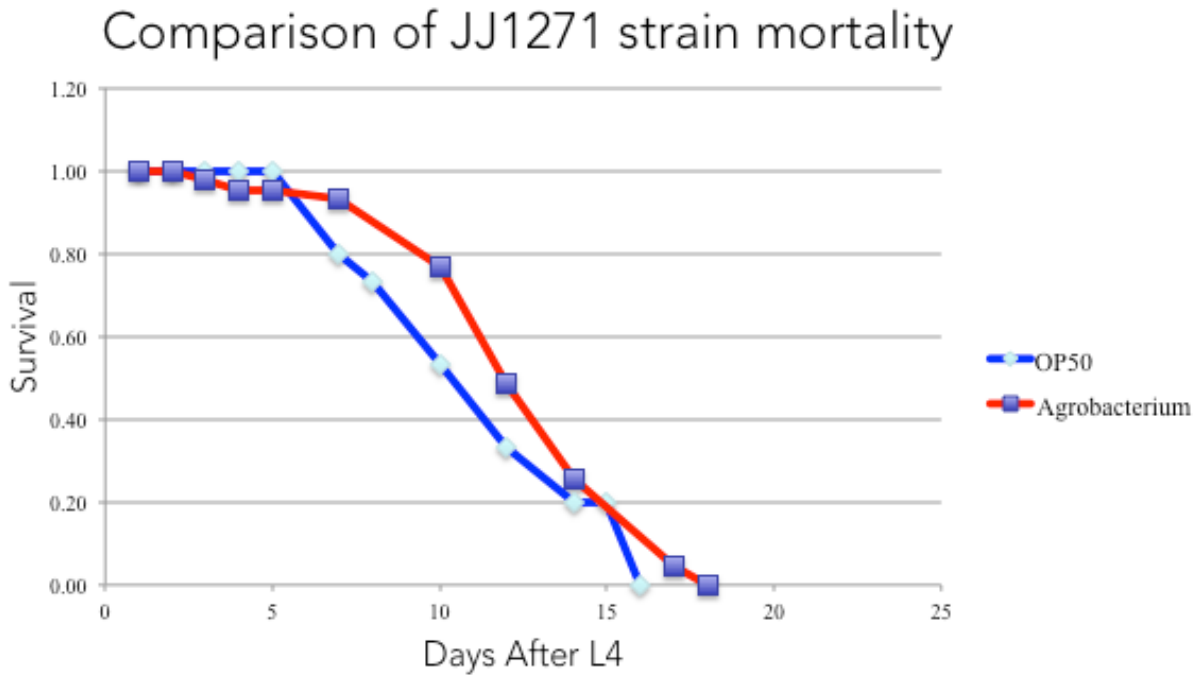

Figure 11: Comparative kill curve of the mutant strain JJ1271 on both *E. coli* OP50 and *Agrobacterium* plates (replotted data from Figures 7 and 8).

Discussion and Conclusion

After observing the fluorescence phenomenon, we hypothesized that the fluorescence in the intestinal cells was caused by *Agrobacterium*, and that even in *glo* mutants (lack autofluorescent granules in intestinal cells), *Agrobacterium* would still cause the same event. Experiments with wild-type worms and *glo* mutants supported the hypothesis and also defined the consequence of the interaction of *C. elegans* and *A. tumefaciens.* In viability, wild-type worms appeared to be more sensitive to the virulence of *Agrobacterium*, but the six-day difference in survival was deemed insignificant using the log rank test. The log rank test is a statistical hypothesis test used to test the null hypothesis that there is no difference between the population survival curves (Bewick *et al.,* 2004). The chi-square for these values was 0.531 and the p value was 0.466. For the difference to be significant, the p value should be less than 0.05. Therefore, the difference is not significant.

The mutants seem to have almost the same response to either bacterium. A reasonable and possible explanation for this is that absent *glo-1* and *glo-4* genes, the mutant strains have reduced and unbiased viability. As discussed above, *glo* genes are required for biogenesis of lysosome-related gut granules. The gut granules are necessary for zinc storage, detoxification and mobilization (Roh *et al.*, 2012). It is a possibility that *glo* mutants experience problems with zinc levels and could ultimately lead to their reduced life span. Zinc deficiency is associated with genetic diseases caused by mutations of zinc transporters, while excess zinc is also deleterious, since it may displace other trace metals or bind low affinity sites, leading to protein dysfunction (Roh *et al.*, 2012).

Growth of the mutant strain JJ1271 presented an interesting case. It exhibited a steady growth rate on both *E. coli* and *Agrobacterium.* However, compared to the other strains when

inoculated with *E.* coli OP50, its survival was about 5 days short. Its survival curve was compared to that of the wildtype strain using the log rank test. The chi-square for these values, which yielded 2.696, gave a p value of 0.101. Again, the difference is not significant.

Experiments conducted have demonstrated that *Agrobacterium*, when inoculated with *C. elegans*, cause intestinal cells and gut cells to fluoresce. However, this phenomenon is not directly linked to the two *glo* genes tested in the experiments because in their absence, the fluorescence persisted. Moving forward, other genes expressed in the intestinal cells will be included in future experiments. There are other *glo* genes (*glo-2* and *glo-3*) that should be considered. It is likely that *Agrobacterium*'s interaction with the intestinal cells trigger or enhance the autofluorescence expressed by the *glo* genes. If this were the case, we would need to test for redundancy among the *glo* genes.

Agrobacterium is a natural pathogen of *C. elegans*. In an experiment to study diverse bacteria and their interaction with *C. elegans*, Couillault and Ewbank found that worms grown on strains of Agrobacterium tumefaciens CFBP 2413 showed a significantly decreased survival (Couillault *et al.*, 2002). This datum coupled with the virulent nature of *Agrobacterium* on its host plants strongly suggest that the *Agrobacterium* diet causes *C. elegans* to have afflicted lives. This withstanding, an *Agrobacterium* diet offers different nutritional value different from *E. coli.* This suggests that the difference in the quality of life can be attributed to the difference in diet. To test this in the future, an experiment with heat-killed bacteria would exclude pathogenic effects on *C. elegans* and reveal the effects of diet change.

References

Bewick V, Cheek L, Ball J: "Statistics review 12: survival analysis." *Critical Care* 2004, 8(5): 389–394

Couillault, C., and J. J. Ewbank. "Diverse Bacteria Are Pathogens of Caenorhabditis Elegans." *Infection and Immunity* 70. (2002): 4705-707. Print.

Hermann, G.J., Schroeder, L.K., Hieb, C.A., Kershner, A.M., Rabbitts, B.M., Fonarev, P., Grant, B.D., and Priess, J.R. (2005). "Genetic analysis of lysosomal trafficking in *Caenorhabditis elegans*." *Mol Biol Cell* 16, 3273-3288.

Praitis, Vida, Elizabeth Casey, David Collar, and Judith Austin. "Genetics." *Creation of Low-Copy Integrated Transgenic Lines in Caenorhabditis Elegans*. N.p., Mar. 2001. Web. 01 Mar. 2013.

Rieckher, Matthias, Nikos Kourtis, Angela Pasparaki, and Nektarios Tavernarakis. "Transgenesis in Caenorhabditis Elegans." *Methods in Molecular Biology* 561 (2009): 21-39. Print.

Roh HC, Collier S, Guthrie J, Robertson JD, Kornfeld K. "Lysosome-related organelles in intestinal cells are a zinc storage site in *C. elegans*." *Cell Metabolism* (2012) 15: 88–99

Tzfira T., Jianxiong L., Benoıˆt Lacroix, and Citovsky. "Agrobacterium T-DNA Integration: Molecules and Models." *TRENDS in Genetics* 20 (2004): 375-383.

Zupan, R., and Zambryski, P. "Transfer of T-DNA from Agrobacterium to the Plant Cell." *Plant Physiology* 107 (1995): 1041-047. Print.